NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION, AND REPAIR
Annual Technical Report
1994

Submitted by

Nicolas G. Bazan, M.D., Ph.D.
Project Director

Period Covered: 20 September, 1993, through 19 September, 1994

Cooperative Agreement DAMD17-93-V-3013

between

United States Army Research and Development Command
(Walter Reed Army Institute of Research)

and

Louisiana State University Medical Center
Neuroscience Center of Excellence

Vision, Laser Eye Injury, and Infectious Diseases

Project Directors
Herbert E. Kaufman, M.D.
Roger Beuerman, Ph.D.

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6. AUTHOR (S)
Nicolas G. Bazan, M.D., Ph.D., Program Director
Director, LSU Neuroscience Center
Professor of Ophthalmology, Biochemistry and Molecular Biology and Neurology

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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2020 Gravier Street, Suite B
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P. O. Box 12211
Research Triangle Park, NC 27709-2211

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13. ABSTRACT *(Maximum 200 words)*

The LSU Neuroscience Center is a comprehensive, multidisciplinary, and transdepartmental entity that unites fundamental neurobiology and the clinical neurosciences in the common goal of elucidating the workings of the brain and contributing to the treatment of currently incurable diseases of the nervous system. The objective of the present program is to find solutions to neuroscience-related problems of interest to the U.S. Army Medical Research and Development Command. The program is focused on exploiting novel neuroprotective strategies that lead to prevention of and repair after neural injury. Converging approaches using state-of-the-art tools of cell biology, neurochemistry, neuroimmunology, neurophysiology, neuropharmacology, molecular biology and virology are proposed. Over the next four years, this program aims to: 1) carry out seven research projects in the basic and clinical neurosciences; 2) expand central, shared facilities with the addition of highly specialized instrumentation not currently available to our scientists; 3) develop laboratory space to permit the physical consolidation and coordination of this research effort; and 4) institute a coordination unit to monitor, facilitate, and administrate the cooperative research programs, as well as to meet the associated budgetary, human resources, facilities, and communications needs for the attainment of the proposed program goals.

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This Technical Report covers the progress made in the first year of this Cooperative Agreement in one project of the original proposal. We hope that this format of the report will facilitate its handling. The table of contents for all the projects has been included in each volume as well as letters from members of the External Advisory Committee of the LSU Neuroscience Center who have conducted an initial review of the work done supported by this Cooperative Agreement.

Nicolas G. Bazan, M.D., Ph.D.
Director, LSU Neuroscience Center
Program Director, USAMRDC Cooperative Agreement

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Abstracts: Psychoneuroimmunology Research Society

1. HSV-1 latently-infected mice display an altered response to stress: Implications for antiviral immunity.
2. Mouse lymphocytes express an orphan opioid receptor
3. Morphine suppresses peritoneal and splenic CTL activity in a dose-dependent fasion in alloimmunized mice
4. The frequency of exposure to morphine differentially affects CTL activity in alloimmunized mice.

Manuscripts:

1. Carr DJJ, Carpenter GW, Garza HH, Baker ML, Gebhart BM (in press) Cellular mechanisms involved in morphine-mediated suppression of CTL activity. In: *The Brain Immune Axis in Substance Abuse* (Sharp, Friedman, Maddin and Eisenstein, eds), Plenum Press.
2. Carpenter GW and Car DJJ (submitted) Pretreatment with β-funaltrexamine blocks morphine-mediated suppression of CTL activity in alloimmunized mice.
3. Carr DJJ and Carpenter GW (submitted) Morphine-induced suppression of spenic CTL activity in alloimmunized mice is not mediated through a δ-opioid receptor.
4. Carpenter GW, Garza HH, Gebhardt BM, Car DJJ (in press) Chronic morphine treatment suppresses CTL-mediated cytolysis, granulation and cAMP responses to alloantigen

"Neurochemical Protection of the Brain, Neural Plasticity and Repair" . . . . .

Project Director: Nicolas G. Bazan, M.D., Ph.D.

Participating Scientists: Geoffrey Allen, Ph.D.
Gary D. Clark, M.D.
Victor Marcheselli, M.S.
John Hurst, Ph.D.
Leo Happel, M.D.
Walter Lukiw, Ph.D.

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Poster presented at the Acoustic Society of America: Kujawa SG, Fallon M, Bobbin RP (1994) A suppressive "off-effect" in the f_2-f_1 DPOAE response to continuous moderate level primary stimulation.

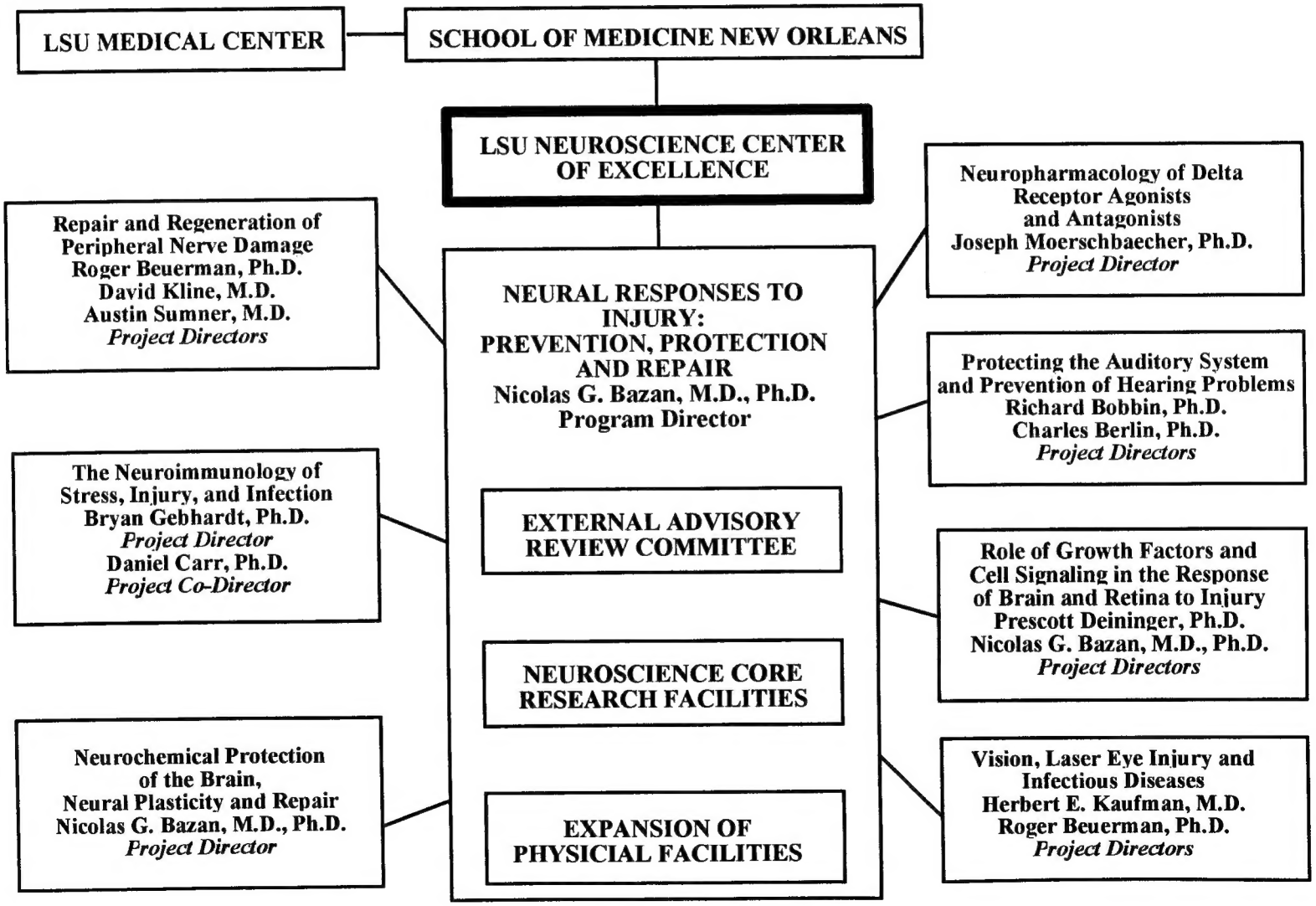
Additional figures for the animals studies

Figures for the human studies

Manuscript: Berlin CI, Hood LJ, Hurley AH, Wen H, and Kemp DT (submitted) Binaural noise suppression linear click-evoked otoacoustic emissions more than ipsilateral or contralateral noise.

Cooperative Agreement Between the US Army Medical Research and Development Command and The LSU Neuroscience Center of Excellence

DAMD17-93-V-3013 20 September, 1993 - 19 October, 1997 $13,860,000



SCHOOL OF
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Louisiana State University
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Neuroscience Center
Office of the Director

19 October, 1994

Commander
U.S. Army Medical Research and Development Command (USAMRDC)
ATTN: SGRD-RMI-S
Fort Detrick
Frederick, MD 21702-5012

Re: Annual report, Cooperative Agreement No. DAMD17-93-V-3013
Neural Responses to Injury: Prevention, Protection, and Repair

Dear Sir,

Please find enclosed the original and five copies of the first annual report for the Cooperative Agreement, referenced above, between the USAMRDC and the Louisiana State University Medical Center School of Medicine, Neuroscience Center of Excellence. This report represents the research carried out during the first year of this agreement (20 September, 1993, to date). It is organized per project, each corresponding to a chapter of the original application.

In addition to the research conducted in the first year of this agreement, the planning for the two additional floors of research space which are to be added to the Lions/LSU Clinics Building, 2020 Gravier Street, New Orleans, LA, has been completed, including all specifications necessary for the start of bidding. Enclosed is one copy each of the program manual (1 vol.) and the project manual (3 vols.) which has been generated by Cimini, Meric and Duplantier, Architects and Planners, for bidding purposes. It should be noted that there will actually be three floors constructed in this one project, two as funded by this Cooperative Agreement and one which is funded by LSU to be used by the School of Medicine for other purposes.

As planned, I arranged to have three meetings between the LSU investigators and their counterparts in the Army to provide program briefings for the work that they were planning to conduct under this agreement as well as to exchange ideas and information of mutual interest. The agendas for each of these meetings are enclosed. These provided both the LSU scientists and those of the Army the opportunity to discuss the work being done, the direction, and the significance to problems of interest to the Department of Defense.

On 2 December, 1993, several of our investigators, excluding the Auditory and Laser/Vision groups, met at the Walter Reed Army Institute of Research, Washington, D.C., with Drs. Frank Tortella, Joseph Long, Mark DeCoster and Jit Dave. These discussions revolved around the neurochemical and neuropharmacological aspects of the program project and provided a forum for the Army scientists tobegin interactions and exchange of information with our investigators.

On 31 January, 1994, the LSU auditory physiology group, represented by Drs. Charles Berlin and Richard Bobbin, and I met at Fort Rucker, AL, with Dr. Kent Kimball and Dr. Ben T. Mozo. These meetings involved presentations and discussions about the protection of the auditory system and prevention of hearing problems in humans.

The LSU investigators involved with the vision research, composed of Dr. Herbert Kaufman, Dr. Roger Beuerman and myself, met on 7 February, 1994, at Brooks Air Force Base, San Antonio, TX. These scientists and those of the Ocular Hazards Research Unit of the US Army Medical Research Detachment made presentations and conducted discussions focused on protection from, repair of, and prevention of laser injuries, specifically to the eye. Each of these information exchanges provided very useful direction and advice for the LSU investigators. These workshops will be conducted annually for the term of this agreement.

At the end of the first year of this program, as planned, I requested that two of the members of the External Advisory Committee of the LSU Neuroscience Center, Dr. Dennis W. Choi, Jones Professor and Head of the Department of Neurology, Washington University School of Medicine, and Dr. Fred Plum, Anne Parrish Titzell Professor and Chairman of the Department of Neurology, Cornell University Medical College, provide a critical review and a written report of the progress of the research accomplished under this Cooperative Agreement. Dr. Choi was given a copy of this annual report and subsequently made a site visit on 15 September, 1994, to the LSU Neuroscience Center. (The agenda for his meeting is attached.) At that time he met with a number of the investigators and administrators involved with whom he discussed many facets of the research being performed under this Agreement. His opinion of the work being done is attached.

Dr. Fred Plum made a site visit on 26 September, 1994, having also been provided previously with a copy of this annual report. He was also given the opportunity to examine the research and other progress made under this agreement and his written critique is also attached. Please note that, near the end of his letter (bottom of page two, first four paragraphs of page 5), Dr. Plum also included a description of projects not directly supported by the Cooperative Agreement but which are very positively impacted by any support of Neuroscience projects. The

reviewers were very complimentary of the positive consequences resulting from this support.

We are very pleased with the progress that has been made. We would like to thank you for the assistance you have given us. Please let me know if there is any further information that I can provide you.

Sincerely,

Nicolas G. Bazan, M.D., Ph.D.
Villere Professor of Ophthalmology,
Biochemistry and Molecular Biology,
and Neurology
Director, LSU Neuroscience Center

NGB/eht
enclosures

JOINT WORKSHOP ON "NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION AND REPAIR"

Sponsored by the LSU Neuroscience Center and Walter Reed Army Institute of Research, Department of Medical Neurosciences

December 2, 1993
Building 40, Room 2133

"Overview of LSU Program" **N. Bazan**	9:00
"Repair and Regeneration of Peripheral Nerve Damage" **R. Beuerman, D. Kline, J. England**	9:20
"The Neuroimmunology of Stress, Injury and Infection" **D. Carr**	10:10
Break	10:20
"Neurochemical Protection of the Brain, Neural Plasticity and Repair" **N. Bazan**	10:40
"Neuropharmacology of Delta Receptor Agonists and Antagonists" **J. Moerschbaecher**	11:15
"Stress and the Dopamine System" **J. Rao**	11:45
Box Lunch Served ($2.00 each)	12:00
"Role of Growth Factors and Cell Signaling in the Response of Brain and Retina to Injury **N. Bazan and J. Cook**	12:10
"An Overview of Neuropharmacology Research at WRAIR on Nervous System Injury and Protection" **Frank Tortella**	13:00
"Animal Models of Spinal Cord Injury and Mechanisms of Blood Flow Changes" **Joseph Long**	13:30
"Evaluation of Excitatory Amino Acids in Neuronhal Cell Culture" **CPT DeCoster**	13:50
"Molecular Biology of Nervous System" **Jit Dave**	14:10
Overall Discussion	14:30
Adjourn	15:00

zwp51\army\workshop.al

Joint Workshop on Neural Responses to Injury:
Prevention, Protection and Repair
Walter Reed Army Institute of Research, Dept. of Medical Neuroscience
U.S. Army Aeromedical Research Laboratory, Fort Rucker, AL
SCHEDULE FOR JANUARY 31, 1994

January 30
12:00 PM - depart New Orleans by car
Hotel: **Comfort Inn, 615 Boll Weevil Circle, Enterprise, AL 36330**
Tel. 205-393-2304, Fax. 205-347-5954

January 31
Visiting - Dr. Kent Kimball, Director, Plans and Programs, USAARL
Dr. Ben T. Mozo, Research Physicist, USAARL
Fort Rucker, AL 36362-5292
Tel. (205) 255-6917, Fax. (205) 255-6937

9:00 AM - Welcome
9:20 AM - Overview of LSU Program - **Nicolas G. Bazan**
9:45 AM - Protection the Auditory System and Prevention of Hearing Problem via Efferent Activation in Humans - **Charles Berlin**
10:30 AM - Break
11:00 AM - Prevention of Hearing Problems in Animals - **Richard Bobbin**
12:00 PM - General Discussion and Lunch
13:00 PM - Adjourn

OCULAR HAZARDS RESEARCH
U.S. ARMY MEDICAL RESEARCH DETACHMENT
7914 A DRIVE (Bldg 176)
BROOKS AIR FORCE BASE, TEXAS 78235-5138

February 7, 1994

Leave New Orleans on Continental flight #1445 at 6:00 PM, arrive San Antonio on Continental flight #1120 at 8:53 PM.

Hyatt Regency San Antonio
123 Losoya St., San Antonio, TX 78205
Confirmation #HY0000605552

February 8, 1994

8:30 *Overview of USAMRD program*
Bruce Stuck, Director, USAMRD

8:45 *Review of Accidental Laser Exposures and Human Tissue Response*
Donald Gagliano, Commander, USAMRD

9:00 *Overview of LSU Program*
Nicolas G. Bazan, Director, LSU Neuroscience Center

9:10 *The Program: Vision, Laser Eye Injury, and Infectious Diseases*
Herbert Kaufman, Chairman, Ophthalmology Dept. LSU

10:00 *Confocal Approach to Cellular Reactions in Wound Healing and of the Lamina Cibrosa.*
Roger Beuerman of the LSU Neuroscience Center

10:30 **BREAK AND LAB TOUR**

10:50 *Neurochemical Protection of the Brain, Neural Plasticity, and Repair*
Nicolas Bazan, Director, LSU Neuroscience Center

11:40 *Basic Fibroblast Growth Factor (bFGF) Treatment of Laser-Injured Retina*
Steven T. Schuschereba, Chief, Biology Section, USAMRD

12:10 *Role of Growth Factors and Cell Signaling in the Response of Brain and Retina to Injury: Focus on the Retina*
Nicolas Bazan, Director, LSU Neuroscience Center

12:50 **LUNCH**

2:50 Depart San Antonio on Southwest flight #803

5:55 Arrive New Orleans on Southwest flight #1055

LETTERS FROM MEMBERS OF THE EXTERNAL ADVISORY COMMITTEE

WASHINGTON UNIVERSITY SCHOOL OF MEDICINE
AT WASHINGTON UNIVERSITY MEDICAL CENTER

NEUROLOGY

Dennis W. Choi, M.D., Ph.D.

Andrew B. and Gretchen P. Jones Professor and Head
Neurologist-in-Chief, Barnes Hospital

17

October 17, 1994

Nicholas G. Bazan, MD, PhD
Director, LSU Neuroscience Center
School of Medicine in New Orleans
Louisiana State University Medical Center
2020 Gravier Street, Suite "B"
New Orleans, LA 70112-2234

Dear Nick:

Thank you for the invitation to visit LSU on September 15 and review early progress made under the LSU Neuroscience Center of Excellence Cooperative Agreement with the U.S. Army Medical Research and Development Command.

You have assembled an impressive array of faculty researchers to study diverse aspects of nervous system injury. Overall, I find the individual projects to be thoughtful and well chosen. With you as director, I am sure that they will be most ably integrated. Your project 3 "Neurochemical Protection of the Brain, Neuroplasticity and Repair" is in my view the clear focal point of the overall program. The identification of new PAF antagonist drugs capable of regulating excitatory synaptic transmission and excitotoxic central nervous system injury, is an attractive and attainable goal. The novel pharmacology theme is also well developed in Dr. Moerschbaecher's Section 4 "Neuropharmacology of Delta Receptor Agonist and Antagonist". Involvement of clinician-investigators in clinical departments, such as Dr. Sumner in Project 1 or Dr. Kaufman in Project 5 are strengths of the program that will enhance its ability to identify human therapeutic interventions.

Progress in the first months of operation appears to be on target. Substantial synergy can be expected between the research programs specifically outlined in this collaborative agreement, and the larger intellectual framework formed the LSU Neuroscience Center of Excellence. Your role as director of both efforts is a vital feature that will ensure maximization of this synergy. In summary, I am most enthusiastic about this LSU-U.S. Army Cooperative Agreement, both for its specific merit and as a prototype mechanism for facilitating effective collaboration between academic and military institutions.

Best regards.

Sincerely,

Dennis Choi

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660 South Euclid Avenue
St. Louis, Missouri 63110
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525 EAST 68th STREET NEW YORK N Y 10021

THE NEW YORK HOSPITAL-CORNELL MEDICAL CENTER

FRED PLUM, M D , CHAIRMAN
ANNE PARRISH TITZELL PROFESSOR OF NEUROLOGY
CORNELL UNIVERSITY MEDICAL COLLEGE
NEUROLOGIST-IN-CHIEF
THE NEW YORK HOSPITAL- CORNELL MEDICAL CENTER
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September 28, 1994

Nicholas G. Bazan, M.D., Ph.D.
LSU Neuroscience Center
2020 Gravier Street
Suite B
New Orleans, LA 70112-2234

Dear Dr. Bazan:

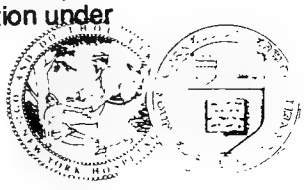
I am pleased to submit this reviewer's report of a Cooperative Agreement between the LSU Neuroscience Center and the US Department of the Army entitled, "Neural Response to Injury: Prevention, Protection and Repair" (henceforth designated as "Injury Study"). The agreement will span four years of effort by the LSU Center; this report describes progress obtained during its first year, extending from September 1, 1993 to August 31, 1994.

Nicholas G. Bazan, M.D., Ph.D. both directs the LSU Neuroscience Center of Excellence and serves as the Program Director of the Injury Study. In addition to Dr. Bazan's personal investigative efforts, seven additional study groups are engaged in research directly related to the Injury Study, as indicated in the administrative diagram attached to this report.

Dr. Bazan's outstanding personal and scientific qualities are the two most important factors in assuring the future success of the LSU-U.S. Army Cooperative Agreement. His leadership and intellectual "taste", as well as his joy in and dedication to brain science penetrate every aspect of the LSU Neuroscience Institute. His enthusiasm has spread to infect his colleagues and many other departments of the Medical School with his high scientific standards and integrity. His knowledge suffuses every dimension of basic neuroscience. His diplomacy and gentle handling of his staff creates their huge loyalty. His energy is contagious. Furthermore, he has the wonderful quality of scientific generosity: always ready to help and encourage others, he is entirely responsible for the continuously improving quality of young persons who are coming to LSU to learn and do important neuroscience.

In addition to the above, Dr. Bazan's specific research is internationally recognized as being of the highest caliber. His personal research contributions to the Injury Study during the past year reflects these high qualities in several ways. They have been published in the most competitively prestigious biomedical research journals. They also add new understandings to both the normal and potentially abnormal effects of the platelet-activating factor (PAF). PAF already is known to be a potent mediator of inflammatory and immune responses. What Bazan and his team now have found is that in low concentrations, PAF transmission may enhance memory and repair mechanisms in brain. Alternately, if released in excessively large concentrations or in combination with certain other molecules, PAF appears capable of causing immune-related tissue damage such as occurs with intense inflammation and/or the induction of genetic prostaglandin synthesis, a step that also may injure brain tissue. This fundamental research emphasizes the complexity and often bidirectional responses that may occur when injury strikes the brain. The results are important and illustrate the difficulties which must be overcome in establishing prevention, protection and repair of brain injuries.

Drs. Bazan and Prescott Deininger have succeeded in developing a series of transgenic mice expressing a dominant mutant of platelet derived growth factor (PDGF). Remarkably enough, the animals thus far have shown no major behavioral alteration under



normal developmental conditions. Their reaction to ischemia, seizures and other circumstances has not yet been tested.

Let me turn now to some of the other, supporting projects: **Drs. R. Benerman, D. Kline and A. Sumner** have made good progress in their studies of neurotrophic factors and other mechanisms in human and experimental neuromas resulting from blunt and crush nerve injuries. Basic fibroblast growth factor (bFGF) was the most prominent factor found in human post-nerve injury neuromas with other specific factors either absent or reaching only very low levels of concentration. More precisely analytic experiments await the analyses of fresh neuronal material from the experimental preparations.

Drs. Herbert Kaufman and Roger Benerman have made brilliant advances using confocal microscopy to examine the cellular details of the human retina. To a degree never before possible they have safely demonstrated in awake human subjects the acute pathophysiology of laser injuries to cornea and their early transformation into fibroblasts. Detailed identification of anterior chamber cells has been possible and current efforts are underway to examine at great magnification the optic disc itself. Ocular fungus and herpes infections can be identified immediately and without introducing foreign substances against the cornea or into the eye. Application of the tool should have an important place in clinically applied military medicine.

During the past year, the investigators also have pursued their earlier discovery that ambient chilling of monkeys latently infected with H. Simplex induces an acute recurrence of cutaneous herpes. Furthermore, chronic ingestion of the beta blocker, propanelol, has been found to ameliorate or prevent the active recurrence. Clinical trials of this important discovery must be pursued as it has important practical aspects.

During the year, the necessary work to establish and equip the glaucoma research laboratory was undertaken. Next year's report can be expected to provide research results from that laboratory.

Dr. Joseph Moerschbaecher and his colleagues in pharmacology have initiated preliminary studies on the influence of delta opoid agonists-antagonists on learning and antinociception. Somewhat surprisingly, the agent damps the CO_2 response of breathing but has no antinociceptive effect. The same investigator is analyzing how anxiogenic drugs affect dopamine neurons in the ventral tegmental area of the rodent brain.

In another preliminary approach, **Drs. H.W. Thompson et al** have initiated experiments passing retroviral gene carriers into the eye with externally applied eye drops, thereby developing a new approach to deliver protection against certain ophthalmologic infections or enhancing the potential success of corneal transplant.

Drs. Richard Bobbin and Charles Berlin, thanks to the DOD grant, have added an excellent postdoctoral student as well as important new equipment to their laboratory. The laboratory's principal subject of interest is to find mechanisms for preventing the audiologic damage produced by intense sound. In guinea pigs, this has been achieved by stimulating calcium-dependent mechanisms in cochlear neurons. In another study, the laboratory has found in human studies that during the delivery of loud, binaural sounds, men and women suppress the noise in opposite sided ears from each other.

The above individual achievements provide only a part of the considerable effort, enthusiasm and success that the U.S. Army grant has brought to the LSU Neuroscience Center of Excellence (NCE). The following steps forward can also be emphasized:

1) Morale in the LSU-NCE rides at high pitch, encouraging scientific collaboration and the generation of new ideas.

2) Funds have been granted to subsidize the necessary equipment and technical personnel to establish a brain bank. Presently, approximately 50 specimens are available in storage with the Center holding good clinical records of the preterminal illness.

3) A program of "starter" grants designed to assist young investigators in conducting merit-deserving, self designed research projects has been initiated.

4) A highly popular state-wide Graduate School outreach summer program has been successfully concluded, attracting a strong interest in neuroscience among gifted college students.

5) An interdisciplinary graduate program in neuroscience was initiated and strongly encouraged by the faculty during 1993-94. As a result, nearly all of the graduate students (including the new entering class) are of very good quality. Indeed, other participating departments say that the Neuroscience graduate students are the best among the LSU biological sciences programs.

Summary. Under the generous auspices of a U.S. Army Cooperative Agreement, the LSU Neuroscience Center of Excellence is not only thriving but headed for far greater future productivity than at any time in the past. The admirable success of the program depends heavily on the foresight, intelligence, creativity and energy of two outstanding scientists, Herbert Kaufman and, especially, Nicholas G. Bazan. Their achievements and those of their colleagues totally warrant continuation of support. Indeed, every indication is that their extramural, non-Army support will continue to grow, making the program stronger and stronger as the years elapse.

One serious problem remains - that of sufficient space in which to do the studies that Dr. Bazan and his colleagues already have conceived so well. Prompt attention to and effective application of must be given to the DOD funds already awarded to construct new research space which will greatly increase the LSU Neuroscience team's opportunities for creative discovery.

I and my colleagues on the External Advisory Board of the LSU Neuroscience Center of Excellence strongly endorse the quality and number of achievements that have come from the U.S. Army-LSU-NCE collaboration. Thanks to strong leadership for the Center and a high degree of internally high morale and interdependence within the Center, it can be anticipated that the Cooperative Agreement will have a major impact on national neuroscience research as well as the specific medical needs of the U.S. Army.

Sincerely,

Fred Plum

Fred Plum, M.D.

FP/moc

VISION, LASER EYE INJURY, AND INFECTIOUS DISEASES

Project Director:

Herbert E. Kaufman, MD
Roger Beuerman, PhD

Participating Scientists:

Claude A. Burgoyne, MD
Emily Varnell
Mandi Conway, MD

FRONT COVER

COOPERATIVE AGREEMENT NO.: DAMD17-93-V-3013

TITLE: Neural Responses to Injury: Prevention, Protection, and Repair

CHAPTER: Vision, Laser Eye Injury, and Infectious Diseases

INVESTIGATOR: Herbert E.Kaufman, M.D., Principal Investigator
Roger W. Beuerman, Ph.D., Principal Investigator

INVESTIGATOR ADDRESS: LSU Eye Center, 2020 Gravier Street, Suite B
New Orleans, LA 70112

REPORT DATE: 1 August, 1994

TYPE OF REPORT: Annual Report - 20 September, 1993, through July, 1994

PREPARED FOR:

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FOREWARD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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(✱) In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources Commission on Life Science, National Research Council (NIH Publication no. 86-23, Revised 1985).

(✱) For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

( ) In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

Investigator Signature

7-26-94

Investigator Signature

7-27-94

ANIMAL USE
20 SEPTEMBER, 1993, THROUGH JULY, 1994

DAMD17-93-V-3013

The experimental animals used during this period for the project, Neural Responses to Injury: Prevention, Protection, and Repair, **Subproject: Vision, Laser Eye Injury, and Infectious Diseases,** are as follows:

Species	Number Allowed	Number Used	LSU IACUC #
Macaca fascicularis	16 monkeys	2	1015
Saimiri sciureus	20 monkeys	2	1049
Mus musculus	270 mice	40 mice	1019

Investigator Signature

7-26-94

Table of Contents

ABSTRACT

A. CONFOCAL MICROSCOPY: For the first time, cellular level observations have been made in the living eye following injury. We found that laser injury to the cornea is first recognizable as an increase in the refractile properties of the nuclei of the cells immediately beneath the injury site. This response, which appears within hours, occurs in cells that eventually become fibroblastic. Thus, the nuclear change may be the earliest predictor of the fibroblastic response. These studies constitute one phase of the confocal project, which was focused on optimizing the optics of the instrument and developing a new mechanical mount that can be used with human as well as animal subjects. Design changes increased the available light and improved image contrast. Observations of human eyes in vivo showed that this confocal microscope can be used on an "as needed" basis and that scanning through the depths of the tissues is rapid and without undue stress to the subject. Animal studies showed that the confocal microscope can be used to repeatedly return to a particular site within the eye in order to follow changes over several weeks. Increased light scattering in ocular tissues or media, as may occur after injury, often limits the type of information that can be obtained using current diagnostic tools such as the slit lamp biomicroscope. With the confocal microscope, however, objective information concerning infectious and inflammatory events at the cellular level may be easily obtained to facilitate real-time, noninvasive diagnosis of clinical ocular disease.

B. GLAUCOMA, TRAUMATIC AND NON-TRAUMATIC: We spent the first year of this support establishing our laboratory and acquiring the necessary equipment to evaluate optic disc compliance as a measure of the mechanical behavior of the structural tissues of the optic disc. Initial mechanical testing sessions using the LDT scanning laser ophthalmoscope suggest that variability may be sufficient to allow pixel by pixel mapping of optic disc surface changes.

C. HERPES: We reproduced and refined our model of recurrent herpes in which latently infected monkeys demonstrate recurrences of ocular herpes when subjected to a chilled environment. This is the only model in existence of stress-induced viral recurrence that produces disease. We used the unique biochemistry of the nervous system, which is deficient in cellular thymidine kinase, an enzyme essential to viral replication, in an attempt to prevent general recurrences by inhibiting viral thymidine kinase and, thus, preventing viral reactivation. Previously, we demonstrated the feasibility of this approach with ethynyl thymidine, which is relatively insoluble. Recently we obtained a new drug, HBPG, and studied its pharmacokinetics; the enzyme inhibition is excellent and the drug is available in quantities useful for further studies. We also showed that viral reactivation due to thermal stress can be modulated by beta adrenergic receptor blockers, offering the hope of a relatively easily available and safe treatment for fever blisters and stress-induced viral recurrences, as well as a clue to the pathways inside the neural cell that permit viral reactivation.

A. CONFOCAL MICROSCOPY

Introduction:

The visual organ processes photic information gathered from the environment; however, it is also subject to damage by light. The ability to assess early damage and to determine potential long-term effects is important. Moreover, after damage has occurred, an early evaluation would be useful to determine a course of treatment.

Recently, a unique new instrument, the real-time clinical confocal microscope, has been developed which can provide cellular-level diagnosis of the layers of the eye. The cornea, lens, and vitreous (the space filling the back of the eye and in front of the retina) are all transparent. The retina with its vessels and neurofiber layer is clearly exposed at the back of the eye. However, the usual ophthalmic diagnostic devices, such as the slit lamp, produce images that are degraded in terms of contrast and resolution. The low contrast of the slit lamp is due both to the inherent low contrast of ocular tissues and to the optics of the instrument, which permit the passage of scattered light obtained from above and below the focal point. The optics of the slit lamp also preclude the use of magnifications higher than about 40x. In contrast, the confocal imaging system is able to produce images of ocular tissue with enhanced contrast and resolution by reducing the plane of focused light, compared with that of a conventional optical microscope. This reduces out-of-focus contributions of light to the image and increases resolution in the focal plane. Thus, the use of increased magnification becomes advantageous and, as increased resolution is

obtained, cells and cellular detail become visible. In addition, increased magnification allows resolution of smaller details within ocular tissues and the ability to see small details such as bacteria, fungus, and focal damage may become an important source of diagnostic information.[1-4] For example, the retina, the delicate light-gathering tissue at the back of the eye, can easily be injured by coherent light. The pigment epithelium, the subretinal space, and inclusions or changes in the tissue density (subretinal edema) may be observable and quantifiable with the confocal microscope.

In the past year, significant progress has been made in redesigning the confocal microscope to make it fully compatible for human use and to increase its range of capabilities. The details of patient interaction with the confocal microscope have become standardized. The diagnostic capabilities of the confocal microscope have been explored, and significant progress has been made in developing new guidelines for some disease situations. A new set of optical elements has been included in the confocal microscope to allow visualization of the optic nerve head, and progress has been made in quantification of the topographical features of the optic nerve head. Other studies have shown the feasibility of rapidly quantifying cellular organization in the front of the eye.

Body:

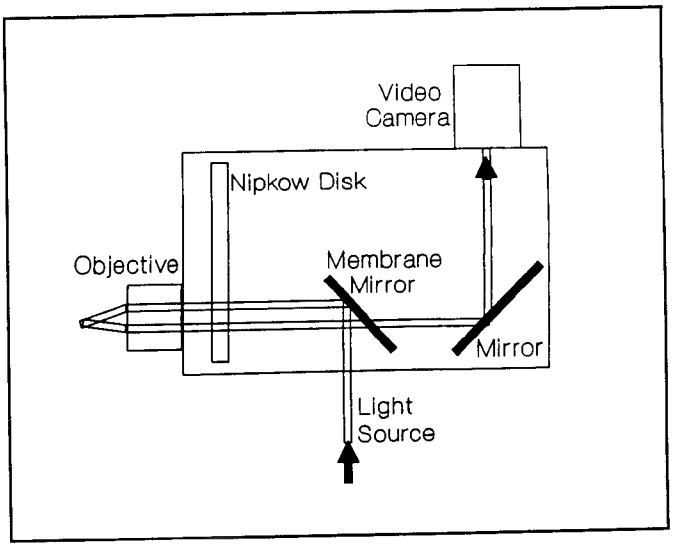
Initially, the design of the confocal microscope was established in a mode that was referred to as the tandem scanning confocal microscope, basically intended for animal research. This design allowed good optical sectioning but had a number of

significant drawbacks. Alignment of the scanning disc was extremely critical, but it was difficult to maintain the alignment of the disc, particularly during use with human subjects. In addition, the disc design was very rigid in that it did not allow exploration of other features and uses of the confocal microscope. This design also had a very low light level that would not permit the use of fluorescent tracers.

These drawbacks have been overcome with a new design – the single-sided confocal microscope. Although refinements are still in progress, the fundamental approach has been now established, a working model has been developed, and initial patient use is underway. Several features of this microscope make it unique. The scanning disc and light path have been redesigned to allow twice as much light through to the objective; early studies indicate that *in vivo* fluorescence microscopy will be possible. Also, the scanning disc can now be removed and replaced in a matter of minutes, and the eye can be observed in a scanning or non-scanning mode. Importantly, this design is self aligning and is robust to movement or contact during use.

The design of this microscope (see figure, next page) is somewhat different, conceptually, from that of the traditional confocal microscope. Light passes from the 100-watt mercury lamp, through a set of pinholes, and into a lens system to the target ocular tissue. Then the light is reflected back from the depths within the target volume at which the lens system is focused. Light passes back through essentially the same set of pinholes and then via a prism to the light detector, which is normally a video camera. The pinholes on the disc are arranged in a spiral or a series of

spirals; there are several sets of pinholes, some combination of which will be used at any one time to transmit and receive light. This system is very different from the scanning system used by a simple scanning device such as a laser scanning confocal microscope, which requires only a single detector for the return light operating in a time sequential fashion. The scanning or Nipkow disc used in the single-sided confocal microscope generates several sets of signals simultaneously as several pinholes are illuminated at any one time, and then data are applied through the surface of the sensor simultaneously. Thus, the real-time features of this microscope are achieved. The basic system uses a sensitive CCD camera to integrate the light over a short period of time, less than 1/30 of a second and, in this way, produces an image that can be displayed on a TV monitor, fed to a digital imaging system, and/or captured on videotape such as a Super VHS tape.



Design of the Confocal Microscope

1) **<u>Macroscopic confocal imaging of the retina and optic nerve head:</u>** The slit lamp is a principal diagnostic instrument in clinical ophthalmology. The magnification of 10 to 40x, a field of 20 mm, and a working distance of 40 mm fulfill the essential requirements for examining the constantly moving patient. Higher

magnifications are seldom used in office practice, although improvements in resolution and contrast are constantly sought.

These improvements have been partially achieved by confocal imaging. However, much of the research has been limited to the use of microscopic lenses, especially immersion lenses. Unfortunately, the small working distance and need for contact with the eye have limited the utility in clinical applications. Thus, this type of instrument is used primarily as an alternative to the specular microscope for corneal imaging and for retinal analysis combined with laser illumination.

In this study we have examined the sclera, cornea, anterior segment, and optic nerve head in rabbit and human eyes using 35-mm camera lenses adapted to the confocal microscope. Retina and vitreous were visualized with a three-mirror Goldmann contact lens. Comparison was made with photographs taken with the slit lamp biomicroscope at the same magnification.

Methods used in this study were as follows. Male Dutch belted rabbits, weighing 2.5 to 3 kg, were used because of the greater pigmentation of the retinal pigment epithelium, simulating that of humans. The rabbits were maintained and handled in accordance with the ARVO Resolution on the Use of Animals in Research. Animals were anesthetized with an intramuscular injection of ketamine HCl (40 mg/kg) and xylazine (5 mg/kg) for *in vivo* examination. Human eyes obtained from NDRI within 24 hours of death were examined by removal of the cornea and lens, leaving the aqueous humor and retina intact. This was possible because the optics of the microscope have been redirected for viewing objects placed vertically, to facilitate

upright human clinical examination. Two objective lenses were used. The lens for macroscopic examination was a Nikkor f = 105 mm, aperture = 2.8, focused at 30 cm. For microscopic examination a Zeiss plan Neofluor 25X/0.8 NA 1 mm water glycerol immersion lens was used. The results of this study showed that the scleral and episcleral blood vessels could be distinguished by focusing on each layer in turn. In clinical applications, this technique may be useful in distinguishing scleritis from episcleritis.

A 25X water immersion lens was positioned 2 mm from the corneal limbus to examine the uveal tract, revealing large choroidal vessels just deep to the sclera. Flow of blood cells through the anastomotic channels could be recorded with a video camera in real time. This technique demonstrates the possibility of *in vivo* evaluation of choroidal perfusion, and may be useful in the clinical evaluation of choroidal infarction and peripheral chorioretinitis.

The cornea was visualized in a single field. Superficial keratectomy wounds were highlighted as if in relief, allowing better assessment of stromal and epithelial wound healing.

The iris pigment epithelium, deep to the stroma, was visible without the need for transillumination, as is required in slit lamp biomicroscopy, to detect pigment dispersion.

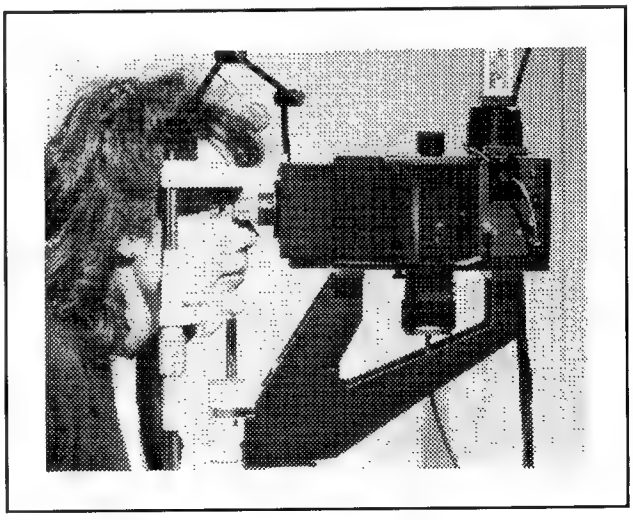
Opacities of the crystalline lens were assessed in serial optical sections. This method may supplant Schleimpflug photography in cataract examination as it permits coronal sections of the lens, with localization of opacities and depth, as well as sector

analysis.

The Goldmann three-mirror fundus contact lens further enabled real-time visualization of the vitreous, retina, and optic nerve head. The hand-held lens, which is used much like the slit lamp biomicroscope, does not compensate for the refraction of the cornea. Optical sectioning of the vitreous is useful in the assessment of vitritis and vitreoretinopathy.

Optic disc analysis, particularly of the neuroretinal rim and peripapillary nerve fiber layer, was improved by the greater lateral resolution of the confocal microscope. Densitometry of the nerve fiber layer was performed from the monochrome photograph of the optic disc; sequential quantitation of nerve fiber loss in glaucoma, as well as other causes of optic atrophy, is also feasible with the use of the confocal microscope.

2) <u>Diagnosis of ocular disease using the confocal microscope</u>: The confocal microscope is a unique tool in the field of ophthalmology. This relatively new instrument uses an incoherent white light which is projected through a spinning disc with thousands of pinholes. In clinical use, the light passes through an applanating objective



Clinical Confocal Microscopy

that focuses the projected light on a specific narrow focal point. The reflected light from structures within the human cornea passes through the objective and back through a sensor. The real time video information is then collected by a CCD camera and stored on super VHS video tape for review. The unique properties of the confocal microscope provide a view of the cornea and other living tissue that was not possible before. Therefore, this microscope has the ability to view both the epithelial and endothelial surfaces of the cornea, as well as Bowman's layer, the stroma, Descemet's membrane, and endothelium. These lateral layers of the cornea have never been viewed *in vivo* before in such a manner.

We have undertaken a study in which we are able to view fungal filaments within a human cornea *in vivo*. Fungus infections of the cornea are extremely threatening to the eye, to vision, and to the visual organ as a whole. Fungus infections are difficult to diagnose; several days may pass before an accurate diagnosis of a biopsy specimen can be made. However, with the confocal microscope fungal elements can be seen throughout the stroma of the living cornea of a patient in real time. Interestingly, the characteristic 45° branching pattern of *Aspergillus* is clearly visible passing practically horizontally through the stroma. *Candida* has been visualized also. *Candida albicans* grows both horizontally and vertically through the stroma of the cornea. We were the first to document observation of this type of differential growth pattern in living tissue, which has both pathologic and therapeutic implications. Also, we have seen inflammatory cells associated with infection, graft rejection, and uveitis. Inflammatory cells are easily visualized as very bright

luminescent bodies on the endothelial surface and within the stroma.

Additionally, we were able to visualize bacteria present in a corneal ulcer and to view *Acanthamoeba* cysts and trophozoite forms in patients with *Acanthamoeba* keratitis. *Acanthamoeba* keratitis is a painful and visually devastating infection, and its accurate and rapid diagnosis is important for therapeutic intervention and the saving of sight. *Acanthamoeba* keratitis occurs almost exclusively among the population wearing soft contact lenses; the prevalence if this disease has increased markedly as the number of individuals wearing soft contact lenses has increased. In the past, diagnosis has relied solely on visualizing the organism in biopsy specimens or cultures, but the success rate of these diagnostic procedures is relatively low. We have shown that both the *Acanthamoeba* cyst and trophozoite form of the organism can be visualized with the confocal microscope.

The advantages of this approach are twofold. First, the diagnosis can be made instantly without waiting for processing of the tissue or growth of the organism in culture; second, the cornea need not be surgically altered as in a biopsy. Thus, the confocal microscope offers a fast, nondestructive diagnostic tool which would speed treatment of these patients and allow follow-up to assess the efficacy of the treatment chosen.

We have enrolled well over 20 patients in our confocal study. None of the patients has exhibited any adverse effects from the confocal microscopy. None of the patients exhibited corneal abrasions, lacerations, or punctate keratopathy. The light from the confocal microscope is dim by comparison with the bright illumination of the

slit lamp and, therefore, poses no problem to the retina or other structures at the back of the eye. In fact, previous work in our laboratory has shown that the confocal microscope is safe for the retina.

It is clear that the confocal microscope is a unique clinical and research tool that offers no known risk to the subject being examined.

3) <u>Confocal microscopy after excimer laser photorefractive keratectomy</u>: The procedure of excimer laser photorefractive keratectomy is currently the most actively investigated refractive surgical technique available for the treatment of myopia. In this procedure, the 193-nm argon-fluoride excimer laser is used to ablate regulated amounts of tissue from the anterior corneal stroma to change the radius of curvature and alter the refractive power. A major postoperative response to the treatment is the development of a subepithelial fibrotic haze, which has been seen in all animals including primates and humans.

In this study we used the scanning confocal microscope to investigate cellular level corneal changes *in vivo* during the first week after photorefractive keratectomy in rabbits. Confocal microscopic images of the cornea provide improved vertical and lateral resolution and image contrast, compared with conventional diffraction with limited imaging methods. It has already been successfully used in the noninvasive investigation of corneal wound healing in radial keratotomy.

In this study, adult New Zealand white rabbits were treated bilaterally with photorefractive keratectomy. The eyes were randomized into four treatment groups:

laser ablations appropriate for 5 diopters, 6 diopters, 7 diopters, and 8 diopters of myopia. The animals were anesthetized with a combination of ketamine and xylazine and were treated in accordance with the ARVO Resolution on the Treatment of Animals in Research.

Quantification of cell number and opacity was accomplished from images of the corneal stroma. Each frame was 400 x 600 microns in size. The quantification of the keratocytes and of fibrosis was performed on the digitized images with the Optimas Bioscan Program on an IBM-compatible computer. Only images at the constant depth of 100 microns from the epithelial surface were used in the measurements.

The results showed that, within 4 hours after the excimer laser procedure, the nuclei of degenerating corneal keratocytes in the superficial stroma had become highly reflective and condensed. While the cells generally retained their parallel orientations, uniformity had decreased. Mild edema contributed to the generalized haze in the otherwise empty background. At 31 hours, cell density was reduced. Surviving cells remained round to oval in shape. By then much of the edema had cleared, coincident with full re-epithelialization. At 2 days after ablation, activated keratocytes were present in abundance. These spindle-shaped cells with dense nuclei were interspersed among accumulations of fibers in the extracellular matrix. Both appeared to be arrayed in parallel lamellae. At 4 days, the distinction between the fibrous and cellular elements had become increasingly blurred. The same was true on day 5, by which time the superficial stroma was dominated by fluffy opacities

and longer fibers. By the end of the first week, the matrix had almost cleared and the pre-eminent feature was interlacing fibrous elements about 100 to 200 microns in length.

Increased depth of ablation was associated with a more protracted initial phase of keratocyte degeneration and a delay in the onset of scar formation. This contrasts with the degenerative changes already well established in corneas with shallow ablations.

After the epithelium healed, it was found to be thicker than it was in the pre-ablated state. The cell size was variable and slightly smaller than before treatment. Superficial cells were also more irregular in shape. Beneath the epithelium of the ablated area, the subepithelial stroma showed dense fibroplasia and scarring. These features were not uniform in distribution, as evidenced by the presence of several large clear lacunae 50 to 100 microns in diameter. Scanning progressively from the anterior to the posterior surface of the cornea showed that the fibroplasia and densities of the extracellular matrix were limited to a layer 100 to 150 microns wide, located beneath the epithelium. In a deeper plane, cell nuclei and spindle-shaped fibroblasts were prominent. However, there was a loss of the dense intracellular matrix seen more anteriorly. The posterior stroma of the treated corneas was essentially identical to that of the untreated corneas. No abnormalities of the corneal endothelium were found, nor were there any detectable changes in Descemet's membrane. These latter observations are unique in that, during this period of time of haze and corneal edema, observation of endothelial cell changes would not be

possible using any other type of ophthalmic instrument except for the confocal microscope.

Conclusions:

We have made substantial design changes both to the optics of the real-time confocal microscope and to its mechanical support for human and animal research. Because the microscope uses only epi-illumination, most of which (about 98%) is blocked by the scanning disk, obtaining 1-2% more illumination will open up exciting new areas of research, such as in vivo fluorescence microscopy. One modification allows the use of standard dichroic filter sets for particular fluorochromes. This approach will be explored in animals to determine the type of material that shows good tissue penetration and produces high levels of fluorescence. The first material to be tested will be labeled lectins, which have been shown to bind to cell membranes and can be used to document changes in membrane structure after wounding. A new type of cell permeant, nucleic acid stains, will also be tested to trace cell movements after injury to the cornea and retina. Some of these binding materials will also be tested for use in detecting bacteria and fungi in the eye. In one planned experiment, bacteria will be fed fluorescein-labeled protein, injected into the eye, then followed by confocal microscopy to determine their fate over a period of several days.

The quantitative analysis of confocal images will also be considered. This process has begun with the demonstration that optic nerve head topography can be quantified in the rabbit from real-time confocal images. A modification of the objective

should allow us to extend this type of observation to the human retina. One advantage of the design of this confocal microscope is that the scanning disk can be quickly removed and replaced, which suggests that it may be feasible to use different disks for specific applications. Examination of the nerve fiber layer after injury may be optimized by a scanning slit disk combined with the new objective currently in development. Other projects involve new approaches to the quantitation of the cellular arrangement of the cornea, which will provide data useful for following the wound response after injury. Specific deconvolution algorithms are being developed to provide greater image clarity. As the processes are dependent on the particular set of optical elements in use, each objective will require individual applications and procedures.

Clearly, the confocal microscope has the ability to track cells after injury and to document the earliest cellular responses to injury, in particular laser injury. Also, predicting the long-term response is made feasible by the ability to return to the same site for observations over time. Another use for this approach will be the study of treatment effects over time in pharmacological studies of potential therapeutic agents in the eye.

References: (*Copies included in appendix)

1. Chew SJ, Beuerman RW, Assouline M, Kaufman HE, Barron BA, Hill JM: Early diagnosis of infectious keratitis with *in vivo* real time confocal microscopy. CLAO J 1992;18:197-201.

CLAO J 1992;18:197-201.

2. Kaufman SC, Beuerman RW, Kaufman HE: Diagnosis of advanced Fuchs' endothelial dystrophy with the confocal microscope. Letter to the Journal. Am J Ophthalmol 1993;116(5):652-653.

*3. Kaufman SC, Chew SJ, Capps SC, Beuerman RW: Confocal microscopy of corneal penetration by tarantula hairs. Scanning. In press.

*4. Chew SJ, Beuerman RW, Kaufman HE: Real-time confocal microscopy of keratocyte activity in wound healing after cryoablation in rabbit corneas. Scanning. In press.

B. GLAUCOMA, TRAUMATIC AND NON-TRAUMATIC

Introduction:

Glaucoma is a disease that involves two separate pathologic processes. In those forms of the disease in which intraocular pressure is elevated, damage to the trabecular meshwork of the eye has occurred. For active duty military, the most important etiologies for this damage are related to ocular trauma and its primary and secondary effects on the trabecular meshwork. Within the retired military population, the separate etiologies contributing to trabecular meshwork damage in chronic open angle glaucoma (the most common form of glaucoma, particularly in the elderly) are most important.

Separate from the pathophysiology of elevated intraocular pressure, the progressive loss of vision that occurs in untreated glaucoma results from damage to the retinal ganglion cell axons and eventual ganglion cell death. While it is possible that damage to the retinal ganglion cells occurs primarily, followed by secondary degeneration of the axons, our best evidence to date suggests that glaucoma or exposure to elevated intraocular pressure causes a progressive optic neuropathy, i.e., the primary site of damage is the ganglion cell axon as it passes through the connective tissue trabeculae of the lamina cribrosa.

The primary focus of this portion of the grant is to study the mechanism of glaucomatous damage to the axons within the tissues of the optic nerve head. Our goal is to use scanning laser ophthalmoscopy of the optic disc surface as well as

confocal microscopy of the lamina cribrosa to study the force distributions, strength, and mechanical behavior of the structural tissues. Our underlying hypothesis is that damage to the structural tissues of the optic nerve head occurs early in the pathophysiology of glaucomatous optic disc damage, and may precede damage to neuronal tissues. Early detection of structural damage may allow intervention to preserve structural tissues, prior to actual loss of axons.

Since arriving at LSU in September, 1993, my efforts have been concentrated on finishing analysis of extensive data acquired during my research fellowship, building a new laboratory at LSU, and preparing for collaboration with Dr. Roger Beuerman for work which will be carried out within our two laboratories. The following is an account of our progress.

Body:

1) <u>Analysis of fellowship data</u>: Since my arrival at LSU, three papers based on work accomplished during my research fellowship with Harry Quigley at Johns Hopkins have been completed. The first, *Global and Regional Detection of Induced Optic Disc Change by Digitized Image Analysis*,[1] describes two image analysis-based strategies for the detection of optic disc surface change. The second, *Clinician Judgment Compared with Digitized Image Analysis in the Detection of Induced Optic Disc Change*,[2] establishes that the small changes in the surface of the optic disc, which we detected by digitized image analysis in paper number 1, are not consistently detected by experienced clinicians viewing photographs of the same

optic discs. The third paper, *Image Analysis Characterization of Global Optic Disc Compliance in the Normal Monkey Eye*,[3] uses the global, image analysis-based, optic disc parameter "mean position of the disc" (MPD) to describe the mechanical behavior of the normal monkey optic disc.

These papers are important because they establish the validity of the techniques we will initially employ in my laboratory at LSU to study the integrity and function of the structural tissues of the optic nerve head. The third paper establishes for the first time that the non-human primate optic nerve head is a compliant structure that behaves mechanically when exposed to acute periods of elevated intraocular pressure. It documents our ability to perform mechanical testing of the optic disc, and establishes the range of normal for a single measure of mechanical behavior: global optic disc compliance.

Data for two additional papers have been analyzed and were presented in part at the Association for Research in Vision and Ophthalmology (ARVO) meeting in May of this year.

Changes in Global Compliance of the Normal Monkey Optic Disc Following the Onset of Chronic Elevated Intraocular Pressure[4] establishes that for the same eyes that were studied extensively as normals, changes in global compliance of the optic disc surface occur very early following the onset of chronic experimental glaucoma. This, too, will be an important paper, in that changes in compliance of the optic nerve head have never before been longitudinally studied following the onset of experimental elevations of IOP. The results provide the first strong evidence for our

hypothesis that damage to the structural tissues of the disc may occur early in the pathophysiology of glaucomatous damage to the disc.

Image Analysis Detection of Fixed Posterior Deformation of the Monkey Optic Disc: A New, Early Finding in Chronic Experimental Glaucoma[5] establishes that, separate from changes in structural tissues as detected by compliance testing, routine longitudinal optic disc imaging that uses our global parameter MPD can detect statistically significant changes in the position of the surface of the optic disc as early as two weeks following the onset of elevated IOP. This provides further evidence for early damage to structural tissues, and will be the first evidence that longitudinal imaging of the optic disc (separate from mechanical testing) with digitized image analysis detects early optic disc change.

2) <u>LSU laboratory start up</u>: In April of this year, we received delivery of the first of two scanning laser ophthalmoscopes from Laser Diagnostics Technologies in San Diego, California (the second will be placed in the clinic so as to begin imaging in humans). Our commitment to this technology represents a change from the Topcon stereoscopic system employed at Johns Hopkins. Scanning laser ophthalmoscopy has several advantages over conventional stereoscopic imaging. First, images can be acquired through small pupils; the inability to do this is an important limitation of stereoscopic systems, which require wide dilation of the pupils for the best images (glaucoma patients are often on medications which cause profound constriction of the pupil). Second, scanning laser ophthalmoscopes gather data from multiple sections of

the tissue. In the case of the LDT instrument, a 256 x 256 pixel grid of intensity data is gathered at 32 progressive depths within the tissues. A separate topographic map of the surface is secondarily generated from the data contained within these 32 sections. While our initial efforts with this instrument will involve only the surface data, we hope eventually to use the data from the 32 sections to do three-dimensional reconstructions of the deeper tissues of the lamina cribrosa and peripapillary sclera. In obtaining this capacity for three-dimensional reconstruction, we believe we will be better able to collaborate with Dr. Beuerman in studies involving the confocal microscope.

Since the arrival of our new instrumentation, we have performed a series of preliminary studies in 2 cynomolgus monkeys in which simplified mechanical testing protocols have been carried out. Early data suggest that, for six images acquired repetitively to characterize the optic disc surface at a single observation point, individual data point variability on a pixel by pixel basis is between 60 and 120 microns. We are awaiting an extensive revision of the operating software which will involve updating the operating system to a Pentium computer with 32 mb of RAM. This update will enable us to rapidly acquire up to 10 images to characterize the disc at a given observation point, and to immediately transfer data to our Silicon Graphics workstation for secondary data analyses. The system upgrade for our laboratory instrument is expected by late August.

We received delivery of our Silicon Graphics Indy workstation in March of this year. This workstation is equipped with a 1 gb fixed disc, 64 mb of RAM, and an

R4400 central processor. In addition to the extensive additional Silicon Graphics software for programming and three-dimensional reconstruction, in May we received the newest version of SAS for IRIX (Silicon Graphics version of a UNIX operating system). Since then, we have hired a computer programmer to begin the process of generating new data management programs within SAS to secondarily process the elevation data from LDT images. Within three months we hope to have finalized the interaction between the LDT instruments in both our research lab and the clinic and our central data processing systems within the INDY.

3) **Preparing for collaboration involving the confocal microscope:** Our laboratory is now located immediately adjacent to the confocal microscopy lab. Dr. Beuerman's Silicon Graphics IRIS computer, our laboratory INDY, the laboratory and clinical scanning laser ophthalmoscopes, and our computer programmer's and biostatistician's PCs are all connected via the Internet. In addition, X-Windows, Exceed 4 Software (Hummingbird) has been installed on a multitude of individual PCs to allow their easy interaction with both the IRIS and INDY workstations. Finally NFS software has been installed on both the IRIS and INDY computers to enable easy and fast transfer of large data sets between them and the laboratory and clinical instruments.

In addition to the two cynomolgus monkeys purchased in March, an additional 11 rhesus monkeys have just been acquired for our first longitudinal study of the non-human primate optic disc in glaucoma. These studies will involve parallel imaging of

the optic disc surface and lamina using the scanning laser ophthalmoscope to image the surface and the confocal microscope to image the lamina cribrosa. We expect studies of the eyes as normals to begin in late fall, and the laser induction of elevated IOP to occur in early 1995.

Conclusions:

From ongoing data analysis of experiments performed at Johns Hopkins:

1) The optic disc in normal monkeys is compliant.

2) Global optic disc compliance is a reproducible test of mechanical behavior of the structural tissues of the optic disc.

3) The compliance of the normal monkey optic disc changes early following exposure to chronic elevated IOP.

4) Damage to structural tissues of the optic nerve head, which can be detected as a change in global optic disc compliance and the onset of fixed posterior deformation of the optic disc surface, may occur early in the pathophysiology of the optic nerve head.

From initial mechanical testing studies utilizing the LDT scanning laser ophthalmoscope at LSU:

1) Variability may be good enough to allow pixel by pixel mapping of optic disc surface change.

References: (*Copies included in appendix)

*1. Burgoyne CF, Varma R, Quigley HA, et al. Global and regional detection of induced optic disc change by digitized image analysis. Arch Ophthalmol 1994;112:261-268.

*2. Burgoyne CF, Varma R, Quigley HA. Clinician judgment compared with digitized image analysis in the detection of induced optic disc change. Ophthalmology (Accepted for publication).

*3. Burgoyne CF, Varma R, Quigley HA, et al. Image analysis characterization of global optic disc compliance in the normal monkey eye. Arch Ophthalmol (submitted)

4. Burgoyne CF, Varma R, Quigley HA, et al. Changes in global compliance of the normal monkey optic disc following the onset of chronic elevated intraocular pressure. (In preparation).

5. Burgoyne CF, Varma R, Quigley HA, et al. Image analysis detection of fixed posterior deformation of the monkey optic disc. A new, early finding in chronic experimental glaucoma. (In preparation).

*6. Burgoyne CF, Varma R, Quigley HA, et al. Image analysis characterization of dynamic optic disc surface deformation in the normal monkey eye. Invest Ophthalmol Vis Sci. 1994; 35 (suppl): 2221.

C. HERPES

Introduction:

Recurrent herpes, both in the eye and elsewhere, is a major problem. There are some 500,000 cases of ocular herpes in the United States each year, and genital and labial herpes also represent a major clinical problem.

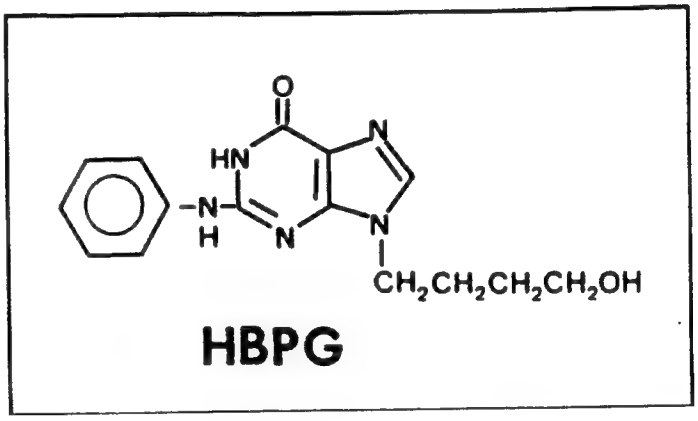
Although systemic antiviral drugs reduce the frequency of genital herpes, they appear to prevent multiplication of virus in the skin so that a sore does not develop. However, they do not prevent the shedding of virus and the infection of partners, and with it, the spread of disease. The present evidence is that systemic antivirals do not prevent recurrences of ocular herpetic disease. We believe that virus reactivates in the ganglion cells and travels to the periphery, where in the genitalia, shedding results; in the eye, however, which is very small, even this amount of virus seems to be enough to produce corneal disease. The goal in both genital and ocular disease is to find a way to stop reactivation in the neural ganglia.

Body:

1) <u>Thymidine kinase inhibition of recurrent ocular herpes</u>: Herpes virus needs phosphorylated thymidine kinase in order to multiply, and so drugs that inhibit viral thymidine kinase were produced. In the periphery, thymidine kinase is available in the cells, but we found that in the central nervous system, the cells have little or no thymidine kinase. In fact, thymidine kinase-deficient viral mutants cannot reactivate

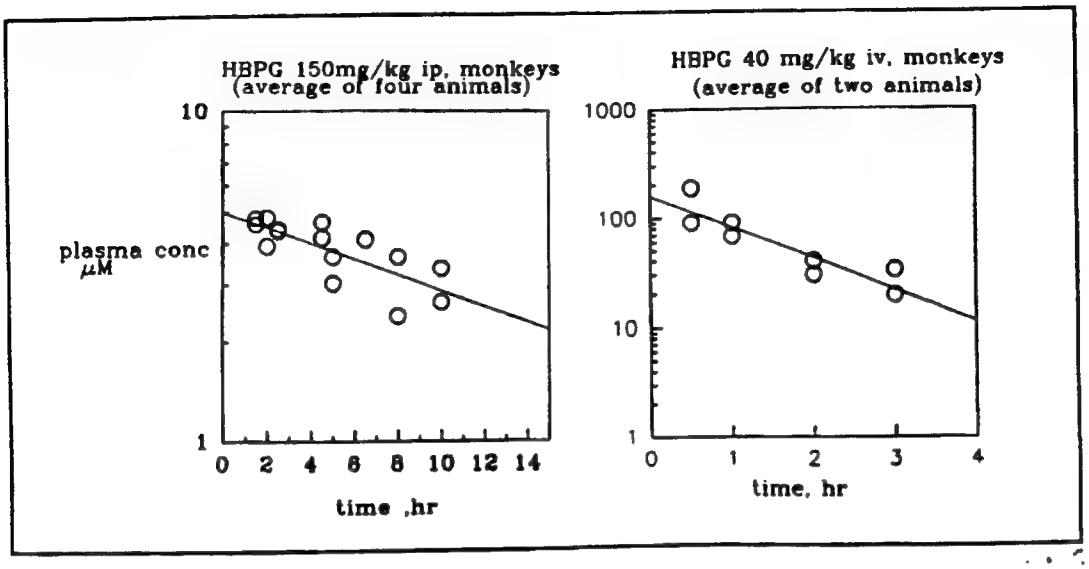
from the ganglion. We tested the first viral thymidine kinase inhibitor (ethynyl thymidine). We studied the enzyme kinetics and the pharmacokinetics of this drug and found them to be suboptimal because the drug is relatively insoluble and the concentration in the serum was marginal. Nevertheless, it seemed worthwhile to attempt a small scale study in vivo. We developed the first model of recurrent ocular herpetic disease in monkeys and found that we could inhibit recurrences of ocular disease with ethynyl thymidine, even though the effect was borderline.[1]

During this past year, we obtained a new drug to test. Dr. George E. Wright of the Department of Pharmacology at the University of Massachusetts Medical School in Worcester became interested in specific viral thymidine kinase inhibitors, in part at our urging. He developed a series of drugs, one of which, HBPG, is a thymidine kinase inhibitor. We have done basic pharmacokinetics and found that HBPG achieves adequate systemic levels, has a half-life of 12.5 hours (as opposed to 4 hours for ethynyl thymidine), and offers the possibility of a useful kind of therapeutic agent. This compound seems to have reasonable solubility, as well as a good partition coefficient and a high affinity constant (K_i = 75 nM), so that a 5 μM concentration should give a 70-fold excess of drug over its K_i. (The effectiveness of the drug depends, in part, on the thymidine



HBPG

concentration in the ganglia, and this concentration has not been measured directly).



Pharmacokinetics of HBPG in Squirrel Monkeys

We did pharmacokinetics in squirrel monkeys, and found a true elimination half-life of approximately 12.5 hours when the drug is given intraperitoneally. Intravenous dosing, however, results in very rapid clearing (2-3 hours), indicating that the drug is not stored in fat. The HPLC analysis of the monkey plasma is so encouraging, that with Dr. Wright, we are preparing to give this drug suspended in corn oil intraperitoneally to squirrel monkeys to determine whether recurrences can be prevented. The method will be essentially the same as that used with ethynyl thymidine,[1] but the monkeys will be subjected to a chill to try to increase the incidence of recurrences.

It seems likely, also, that ribonucleotide reductase inhibitors could be useful to

prevent herpes recurrences since they, too, inhibit an essential specific viral enzyme.[2] To date, those available have too short a half-life to be useful when given systemically, but further work in this area is continuing, and we expect drugs in this family to be available during the tenure of this grant.

2) <u>Beta blocker modulation of viral reactivation</u>: We have hypothesized that one class of mediators that can interact with cells of the nervous system and modulate viral reactivation from latency is that of beta adrenergic receptor blockers. At the time of submission of the original proposal, we had just initiated a series of experiments to investigate this hypothesis. Recently, we have obtained preliminary results regarding the effect of the beta blocker, propranolol, on suppressing stress-induced viral reactivation in mice.

Groups of mice were infected with the McKrae strain of herpes simplex virus type 1 (HSV-1). At 55 days after primary corneal infection, groups of latent mice were subjected to the stress paradigm originally described by Sawtell and Thompson.[3] Each mouse was restrained in a 50 ml centrifuge tube that had 40 holes punctured in its sides so as to permit the exchange of air and water. The mice were immersed up to their necks in 42° C water for 10 minutes, then quickly dried and returned to their cages. Immediately following the application of the stress, the mice were given one intraperitoneal injection of propranolol hydrochloride at a concentration of 1 mg/kg. Twenty-four hours later, the mice were sacrificed, their eyes swabbed for ocular surface culture, and the trigeminal ganglia collected for isolation of infectious virus

and viral DNA. Control groups included latent mice not subjected to stress, but given an injection of propranolol, latent mice that were stressed and given an injection of placebo, and uninfected mice.

Ocular swabs wetted in tissue culture medium were touched to the ocular surface and twirled in individual wells of 24-well culture plates containing monolayers of CV-1 cells. The culture plates were incubated for 21 days and observed daily for cytopathic effect (CPE).

Trigeminal ganglia from each mouse were collected and processed individually. One ganglion from each mouse was immediately homogenized in tissue culture medium, clarified by low speed centrifugation, and the supernatant plated on individual wells of CV-1 cells. These plates were incubated for 21 days and the appearance of CPE recorded.

The other trigeminal ganglion was homogenized in a DNA extraction buffer, and the total DNA (genomic and viral) extracted from each ganglion. This DNA extract was quantitated by spectrophotometry at 260 nanometers and subsequently, viral nucleic acid sequences were amplified using primers for viral ribonucleotide reductase and viral DNA polymerase genes. Following 35 rounds of amplification, the amplified DNA was examined by agarose gel electrophoresis and ethidium bromide staining.

The results of these preliminary experiments are given in the tables below. Tear film ocular swab cultures obtained 24 hours after stress induction were positive in 50% of the samples collected (Table 1). These results indicate that the stress

paradigm induces viral reactivation in a significant percentage of mice. One peritoneal injection of propranolol at the time of stress induction reduced the incidence of viral reactivation to 28% (Table 1). Thus, it appears that the beta blocker may, in some way, modulate reactivation of the virus from latency.

TABLE 1: Ocular Swab Cultures for Infectious Virus	
Treatment Group	Infectious Virus (No. Positive/Total)
Propranolol	4/14 (28%)
Placebo	4/8 (50%)

Homogenates of trigeminal ganglia from each group of mice were observed for their ability to induce CPE for 21 days. We recorded that 50% of these homogenates from stressed, placebo-treated animals yielded CPE on CV-1 cells (Table 2). In contrast, the homogenates from stressed, propranolol-treated animals yielded CPE in 36% of the samples (Table 2). These results suggest that, in peripheral nervous tissues such as the trigeminal ganglion, the beta blocker may suppress viral reactivation.

TABLE 2: Ganglionic Homogenate Cultures for Infectious Virus	
Treatment Group	Infectious Virus (No. Positive/Total)
Propranolol	5/14 (36%)
Placebo	4/8 (50%)

Several other approaches to analyzing and investigating the role of beta blockers in modulating viral reactivation are planned. Molecular biological analysis of expression of viral genes is in progress. In these preliminary experiments, homogenates of trigeminal ganglia were subjected to RNA and DNA extraction, reverse transcription to cDNA, and amplification of the DNA

using the polymerase chain reaction (PCR) in an effort to determine if the beta blocker affects the expression of selected viral genes during viral reactivation. As shown in Table 3 below, there was a difference between our ability to amplify viral DNA from placebo-treated and propranolol-treated stress reactivated animals. Generally amplification of viral DNA from trigeminal ganglia of latent mice yields nothing. The copy number of viral genes in a latent mouse ganglion is sufficiently low as to fail to yield a product following 35 rounds of amplification by PCR. However, following the application of the stressor a high percentage (100%) of the DNA extracts from latent mice contained amplifiable viral DNA (Table 3). In contrast, 36% of DNA samples from the trigeminal ganglia of stressed and propranolol-treated trigeminal mice yielded viral DNA (Table 3).

TABLE 3: Viral DNA in Trigeminal Ganglia in Stressed Mice	
Treatment Group	Viral DNA (No. Positive/Total)
Propranolol	5/14 (36%)
Placebo	8/8 (100%)

Overall, these preliminary experiments suggest that beta blockers may modulate viral reactivation following stress. The role and mechanism of action of the beta blockers remain to be determined. Nevertheless, these results are sufficiently encouraging to warrant further investigation of this class of compounds as part of a potential therapeutic strategy for the prevention of herpes viral reactivation.

Conclusions:

1) The prevention of ocular herpetic reactivation in the ganglia with the use of thymidine kinase inhibitors has been shown to be possible, and improved drugs with greater solubility and the potential for higher serum concentrations are being tested.

2) Stress-induced reactivation of herpes virus from the ganglia in mice was reduced by injection of a beta adrenergic receptor blocker, propranolol, in terms of the percentage of positive ocular swabs, as well as the percentage of ganglia in which herpes virus DNA can be detected by the polymerase chain reaction. This suggests that the beta blocker in some way modulates the reactivation process, thereby preventing shedding and possibly also recurrent disease.

References:

1. Kaufman HE, Varnell ED, Cheng YC, Bobek M, Thompson HW, Dutschman GE. Suppression of ocular herpes recurrences by a thymidine kinase inhibitor in squirrel monkeys. Antiviral Res 1991;16:227-232.

2. Safrin S, Schacker T, Delehanty J, Hill E, Corey L. Potential for combined therapy with 348U87, a ribonucleotide reductase inhibitor, and acyclovir as treatment for acyclovir-resistant herpes simplex virus infection. J Med Virol Suppl 1993;1:146-149.

3. Sawtell MN, Thompson RL. Rapid in vivo reactivation of herpes simplex virus in latently infected murine ganglionic neurons after transient hyperthermia. J Virol 1992;66:2150-2156.

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Real-Time Confocal Microscopy of Keratocyte Activity in Wound Healing after Cryoablation in Rabbit Corneas

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Summary: A modified tandem scanning confocal microscope was used for real-time in vivo examination of the rabbit cornea following a cryogenic injury. The corneas of New Zealand white rabbits were frozen with a probe that had been cooled by immersion in liquid nitrogen, effectively destroying keratocytes in a central 5 mm diameter zone throughout the total thickness of the cornea. In these eyes, keratocyte repopulation and corneal stromal wound healing proceeded similarly to that which occurs after epikeratophakia, a refractive surgical procedure designed to change the curvature and optical power of the cornea. In epikeratophakia, a cryolathed donor corneal stroma lenticule is sutured onto the bare stroma of the recipient cornea. The collagen tissue lenticule is repopulated by keratocytes (corneal fibroblasts) that migrate in from the host cornea. In our study, the confocal microscope permitted sequential, noninvasive examination of the corneal stroma in the treated animals. Necrosis of the keratocytes, followed by activation of the remaining viable cells in the corneal periphery, was observed in the first 2 to 3 days after cryo injury. A fine stromal fibrous network was seen to develop; in three eyes, this network progressed to the development of a retrocorneal fibrous membrane and dense stromal fibrosis, both of which resulted in significant loss of corneal clarity. Our results suggest that the confocal microscope may be a valuable tool to provide much needed information on wound healing processes at the cellular level after corneal surgery and injury.

Key words: confocal microscopy, cornea, cryosurgery, eye imaging, noninvasive imaging, real-time

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Introduction

Since its introduction to ophthalmology by Cavanagh in 1988 (Jester *et al.* 1988), the confocal microscope has been successfully applied to the noninvasive diagnostic evaluation of the cornea in various diseases (Beuerman *et al.* 1992; Cavanagh *et al.* 1990, 1993; Chew *et al.* 1992a,b, 1993; Jester *et al.* 1990, 1992b; Kaufman *et al.* 1993; Masters and Kino 1990) and in wound healing (Jester *et al.* 1992a; Underwood *et al.* 1992). Its chief advantages over conventional in vivo clinical imaging are superior resolution and image contrast, as well as serial optical sectioning ability (Inoue 1992; Koester 1992). We applied this technique to the study of the wound healing response after central corneal freezing injury in the rabbit.

The clinical significance of keratocyte (corneal fibroblast) repopulation of cryoablated corneas is illustrated by the surgical technique of epikeratophakia. In this refractive surgical procedure, first described by Kaufman (1980), the curvature and optical power of the cornea are altered by means of an optically corrected addition made from a cryolathed donor cornea. This lenticule, which consists only of stromal tissue, is sutured onto the patient's cornea after the epithelium is removed. Epikeratophakia has been used to treat high myopia, keratoconus, and aphakia in children. Keratocyte repopulation of the epikeratophakia tissue lens occurs by cell migration from the host stroma after fibroblastic transformation of these otherwise metabolically quiet cells (Klyce and Beuerman 1988). This cellular aspect of the healing process cannot be followed by conventional clinical microscopy; however, it is acknowledged that the repopulation of the collagenous stroma of the epikeratophakia lens is important for the clinical recovery of the optical properties of this prosthetic device. A better understanding of the healing process is thus required. In particular, morphologic studies describing the time course of the keratocyte repopulation in response to the procedure are needed.

Confocal microscopy has been used to study corneal healing in the living eye in other forms of refractive surgery, including radial keratotomy (Jester *et al.* 1992a) and excimer laser photorefractive keratectomy (Cavanagh *et al.* 1993; Lam *et al.* 1993; McDonald *et al.* 1992). In the present study, we have investigated the stromal keratocyte response in vivo after cryoablation. The cryoablation procedure creates a central zone in the stroma that is depleted of keratocytes throughout the

entire thickness of the cornea. In this study, we followed the fibroblastic motile phase of the repopulation of this depleted zone using confocal microscopy over a period of 6 weeks.

Materials and Methods

Animals

Older rabbits of either sex weighing > 4.5 kg were selected for use. The techniques used were in compliance with the ARVO Resolution on the Use of Animals in Research. Twenty New Zealand white rabbits were anesthetized with an intramuscular injection of ketamine hydrochloride (40 mg/kg) and xylazine (5 mg/kg). An eyelid speculum was used to maintain the open eye during the procedure. Cryoablation was carried out following topical anesthesia with proparacaine. The central core of a 5 mm diameter, stainless steel corneal trephine was immersed in liquid nitrogen for 5 min, and then centered over the pupil and applied to the apex of the cornea for 10 s. The extent of freezing was not permitted to reach the iris or the crystalline lens. The cornea was thawed immediately with warm normal saline irrigation and the entire freezing-thawing procedure was repeated. The eyes were examined by means of confocal and slit lamp microscopy daily for 1 week, then biweekly until no further changes in the cornea were observed.

Instrumentation

The eyes were examined with a confocal microscope (Noran, Middleton, Wisc.), specially modified for ophthalmic use. The Nipkow disc with 20,000 60-μm apertures rotated at 500 rpm and illumination was supplied by a 200 W mercury lamp. The optics of the microscope had been redirected horizontally for viewing of seated subjects as required for standard ophthalmic examination. A 25×/0.8 NA water-immersion objective lens (working distance 0.5 mm) was used. Real-time observations were carried out by viewing the video image on a high resolution monitor (Sony Medical Grade). No computer enhancement was required or carried out. Permanent records were obtained by recording on S-VHS tape and hard copies were made with a video printer (Sony UP-5000).

Results and Observations

Normal Areas of Cryoablated Corneas

The substantia propria, or stroma, forms 90% of the thickness of the cornea. It is avascular and consists of collagenous lamellae, 2–5 μm in thickness, interspersed with keratocytes and ground substance (Klyce and Beuerman 1988). The lamellae are broad bands of interlacing collagen type I fibrils extending across the diameter of the cornea and arranged approximately parallel to its surface. The cells of the stroma, the keratocytes, are located along the interface between lamellae, but the lateral processes of these cells extend well out along the interface and into adjacent lamellae on either side of the cells.

By confocal microscopy, keratocytes in the unfrozen areas of the cryoablated eyes appeared as 10–15 μm long, oval-shaped bodies (Fig. 1). Only the nuclei were readily visualized; the cell bodies and cytoplasmic processes were not well resolved. Several cell bodies could be visualized at one plane of focus, suggesting that they were arrayed in parallel with the collagen lamellae. Moreover, the orientation of the ovoid nucleus tended to change with depth in the cornea; however, no attempt was made to correlate the depth and orientation of the nucleus. The structure of the individual lamellae was essentially transparent in the normal cornea. In contrast to normal histologic sections, which are oriented perpendicular to the corneal surface, the confocal microscope provides en face optical sections. This view is shown by the relatively high density of these cells in a particular plane of focus. The cellular structure of normal, untreated cornea in our study was similar to that previously noted by confocal microscopy (Cavanagh *et al.* 1990; Chew *et al.* 1992a).

Keratocyte Degeneration

Within 24 h of the cryoablation, confocal microscopy clearly demonstrated the loss of keratocytes throughout the thickness of the cornea (Fig. 2); the central and anterior portions of the cornea were essentially devoid of cells. Earlier attempts at examination were unsatisfactory because of the dense stromal edema that appeared as an amorphous haze through which no cellular details were discernible. On the first postoperative day, despite persistent mild edema, confocal microscopy permitted visualization of degenerating nuclei throughout the 500 μm thickness of the corneal stroma. The pyknotic nuclei were highly refractile irregular ovoids, or spindle-like bodies, of varying sizes. No increased density of cytoplasmic processes of the keratocytes could be seen. As shown by other studies (Rozsa *et al.* 1983), the lateral processes do become more prominent during wound healing because of the transformation of the cells to a fibroblastic state and an increase in the cyto-

FIG. 1 Normal keratocyte morphology in the rabbit corneal stroma. Only the ovoid nuclei of the cells, but not the cell bodies or extracellular matrix, are seen (confocal microscopy; original magnification, 230×).

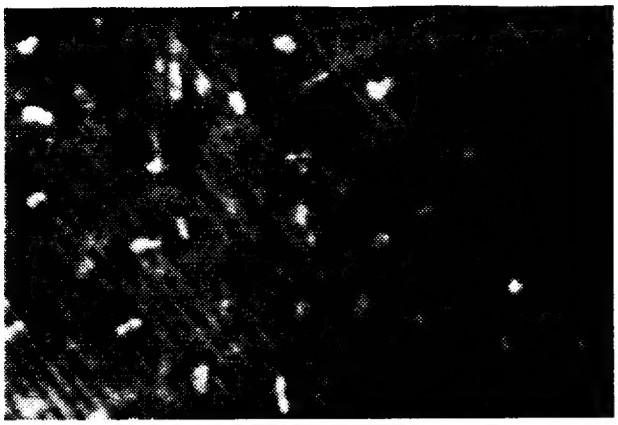

FIG. 2 Twenty-four h after hypothermic injury, degenerating keratocytes are seen as irregularly shaped bodies of variable size that are more refractile than undamaged cells. A damaged stromal nerve is also visible (confocal microscopy; original magnification, 230×).

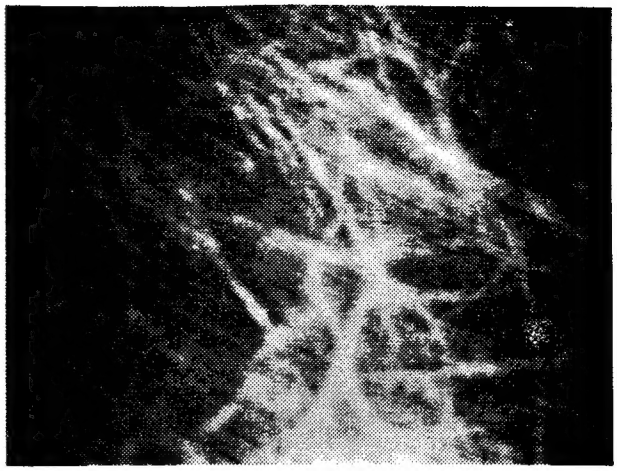

FIG. 3 At 4 days, the fibroblasts are interconnected by a three-dimensional array of 10–20 μm thick fibers (confocal microscopy; original magnification, 230×).

plasmic volume. In addition, the lateral processes have been found to extend for great distances into a central wound.

Keratocyte Regeneration and Fibrils

On the second postoperative day, isolated activated fibroblasts were found around the periphery of the wound. These cells exhibited fine, radiating, cytoplasmic processes. These cells often were found in the subepithelial layer and largely in the periphery in a surrounding zone of less damaged cornea outside the cryoablation.

After 3 to 4 days, the isolated fibroblasts were gradually incorporated into a network of interlacing fibrils (Fig. 3). These extended collateral processes of the keratocytes appeared to take on a more three-dimensional orientation than in the early response after wounding. Processes were found to extend upward to superficial and downward to deeper layers of the stromal lamellae. This intricate morphology was not evident in normal histologic sections, which usually depict only the cell bodies of keratocytes and fibroblasts in any detail. A few distinct bands of refractile material were distinguished in this network; these bands may represent newly synthesized collagen fibrils secreted by the fibroblastic keratocytes. There was a gradient of increasing density of these bands from the deep stroma upward to the subepithelial stroma. At this time, the deep stroma near the endothelium was still devoid of cellular or fibrous elements. The fibrillar web thinned toward the periphery, where only isolated keratocyte nuclei in clear stroma were observed. The main source of keratocytes contributing to the repopulation of the cryoablated stroma was the activation of cells in the periphery of the wound. Occasional cells in this zone showed thin, 2–4 μm thick cytoplasmic processes that were not visible in the normal cornea. No inflammatory cells were detected. It has been shown previously that cells of immune origin in the process of invading corneal tissue from the vascular space are easily visualized by confocal microscopy (Chew *et al.* 1992a).

In half the eyes, fibrous remodeling occurred by 1 week after cryoinjury. This process resulted in a gradual clearing of fibrils from the stroma and a partial return to the nonrefractile background that characterized normal stroma. During this period and in the ensuing weeks, individual fibroblasts were again discernible. These cells showed typical, thin cytoplasmic processes that emanated almost at right angles to the cell body and were in contact with adjacent cells and fibrils. However, in the remaining eyes, fibrous cellular proliferation continued unabated, producing a generalized reticulated haze that was still progressing 2 weeks after freezing (Fig. 4).

By the sixth postoperative week, corneal clarity was almost normal in most eyes. However, a dense fibrocellular scar had formed in the eyes with an exaggerated keratocyte response (Fig. 5). The compact nature of the fibrous tissue prevented resolution of individual keratocytes, and visualization of the endothelium was also impaired.

Retrocorneal Fibrous Membrane

On the tenth day, one eye showed endothelial changes at discrete loci within the zone of cryogenic injury (Fig. 6). Proliferation of the endothelial cells resulted in localized thickening of the normally single-layered endothelium. The retrocorneal membrane extended 20–40 μm into the anterior chamber. While the majority of cells retained their rounded morphology, a significant proportion had undergone fibroplasia and had acquired a spindle-shaped appearance. This phenomenon was especially prominent in the centers of these fibrous foci.

Discussion

Cryotherapy of the cornea destroys the cellular elements—the epithelium, the keratocytes, and the endothelium (Zavala *et al.* 1985). It may also damage the collagen fibrils and lead to edema and vascularization. In addition to the use of cryolathing in the production of keratorefractive lenses, cryotherapy has been advocated for the adjunctive treatment of the trophozoites

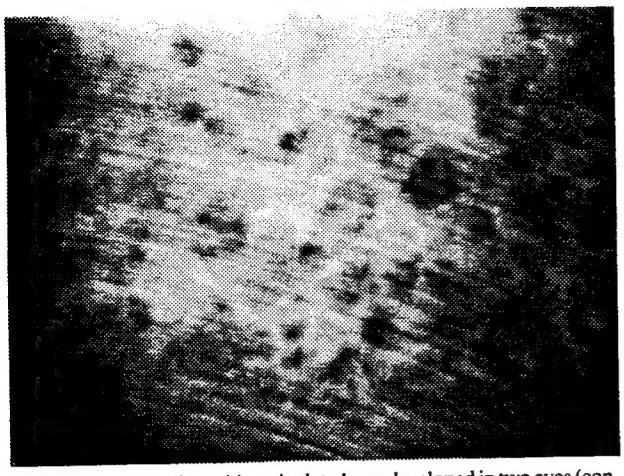

FIG. 4 After 2 weeks, a thin reticulated scar developed in two eyes (confocal microscopy; original magnification, 230×).

FIG. 5 Six weeks postoperatively, a dense, stromal fibrocellular scar was seen in two eyes (confocal microscopy; original magnification, 230×).

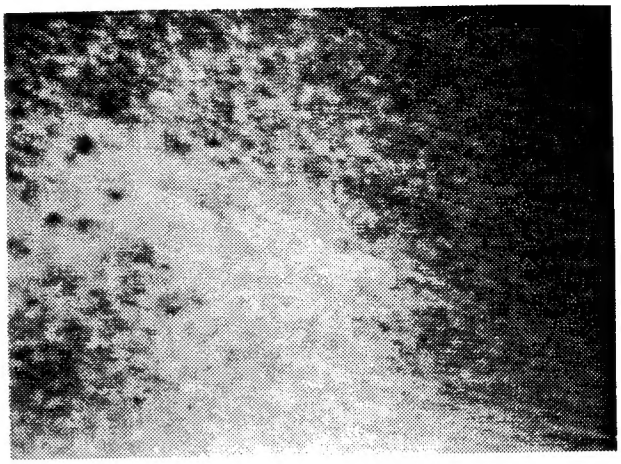

FIG. 6 A retrocorneal fibrous membrane is seen in a single cornea. Fibroblast transformation and fibrous proliferation are found in isolated foci in the endothelium (confocal microscopy; original magnification, 230×).

and cysts in acanthamoebic infectious keratitis (Binder 1989). Surgical full-thickness cryotherapy to a cornea often involves the use of a brass rod dipped in liquid nitrogen or a nitrous oxide cryo unit set at −80°C. Lesser degrees of corneal injury can occur by exposure to cold air or supercooled fluids. All forms of corneal thermal injury pass through an initial phase of tissue destruction, a resting phase, and a period of definitive repair.

The extent of tissue destruction varies with the initial temperature and duration of contact with the cryogenic agent. Minor hypothermic injuries may be limited to the epithelium and cause focal or diffuse loss of superficial or basal cells. They usually are accompanied by transient edema and infiltration of inflammatory cells. Rapid freezing causes solidification and crystallization of intracellular and extracellular fluids. In the process of one or more cycles of freezing and thawing, the changes from solid to fluid cause disruption of epithelial and stromal cell membranes and organelles. Measurement of stromal tissue temperature during application of cryoprobes has demonstrated a direct relationship between the depth of tissue damage and the temperature of the probe. When liquid nitrogen-cooled probes are applied to the corneal surface, they lower the subepithelial temperature to −20°C or lower. This causes necrosis of the epithelium and the stroma; however, the extent of stromal destruction is limited, and the remaining collagen structure serves as a normal scaffold for reepithelialization.

The process of freezing also increases the size and number of spaces between the collagen fibrils (Schanzlin *et al.* 1983). More severe injuries can cause the stromal collagen to undergo varying degrees of necrosis followed by reactive scarring. Wound healing after hypothermic injury is very different from wound healing following penetrating keratoplasty, during which the donor epithelium, stromal keratocytes, and collagen fibrils are undamaged. Thus, cryoablation provides a useful in vivo model for the study of injury-related fibroblast proliferation, migration, and production of extracellular matrix.

Few studies have reported the time course of healing after epikeratophakia or cryosurgery. Many of these investigations are based on static observation (Katakami *et al.* 1991; Unterman *et al.* 1989; Werblin and Klyce 1982). Even activity labeling with radioisotopes is compromised by the in vitro manipulations performed. In the studies of Katakami *et al.* (1991), ^{3}H-thymidine labeling of keratocytes was shown to increase at about Day 16 and peak on Day 28. Histology showed that the population of keratocytes is sparse, with an increased accumulation of keratocytes at the interface between the lenticule and host stroma. Using the confocal microscope, we detected cells within the ablated zone 2–3 days after injury. Also, in the Katakami *et al.* (1991) study, ^{3}H-proline uptake by keratocytes, as a reflection of collagen synthesis, increased at 2 weeks, peaked at 4 weeks, and declined thereafter. These findings contrast with the confocal observations of new collagen formation as early as 1 week after ablation. While the host-graft interface in epikeratophakia (which was absent from our model) induced a time lag in the appearance of repopulated keratocytes and a renewal of their synthetic activity in the cryolathed tissue, we feel that the confocal microscope enhanced our detection of migrating fibroblasts and the production of new collagen.

Interindividual variations in healing rates complicate conventional histologic analysis, requiring a statistical treatment

of pooled data to obtain approximations of the rates and locations of keratocyte and collagen recovery. These problems were resolved by sequential examination of individuals by in vivo confocal microscopy.

Some histologic studies have described the morphology of activated keratocytes (Assouline *et al.* 1992; Klyce and Beuerman 1988). However, an appreciation of the three-dimensional morphology of the activated keratocyte or corneal stromal fibroblast is difficult with such techniques. In addition, the limited resolution of the light microscope limits observation of the fine cytoplasmic fibrils emanating from the injured cells. Our confocal observations clearly demonstrate the abnormal morphology of activated keratocytes and the interaction of the collateral processes with those from cells of adjoining layers. Keratocyte proliferation and repopulation were followed by apparent collagen synthesis by these cells. The confocal microscope may also allow for study of the process of collagen remodeling, without recourse to immunohistochemistry or radioisotope labeling. This would be valuable, as collagen remodeling is the major factor causing scarring and vision loss after surgery. Presumably, confocal microscopy would permit a close follow-up of patients and a timely pharmacologic intervention with steroids or other growth-modulating agents.

It has previously been shown that, under conditions of inflammation, infection, wounding, and atypical repair, corneal endothelium can respond by undergoing fibroplasia (Michels *et al.* 1972). These morphologically transformed cells can produce fibrillar collagen and deposit an abnormal extracellular matrix to form a retrocorneal fibrous membrane. This would impair vision by reducing light transmission and by diffraction. It has been suggested that the transforming growth factor may be a corneal endothelium modulation factor that is derived from polymorphonuclear leukocytes and modulates type IV collagen synthesizing endothelial cells to type I collagen synthesizing cells (Kay *et al.* 1993). However, in this study, we saw no increased density of inflammatory cells in the vicinity of the membrane or in the aqueous humor and/or microkeratic precipitates on the endothelium.

In summary, in vivo confocal microscopy was found to be useful in the evaluation of corneal wound healing following physical injury, readily permitting noninvasive study of the complex processes in the keratocyte response to injury and collagen remodeling of the stroma.

Acknowledgments

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References

Assouline M, Chew SJ, Thompson HW, Beuerman RW: Effect of growth factors on collagen lattice contraction by human keratocytes. *Invest Ophthalmol Vis Sci* 33, 1742–1755 (1992)

Beuerman RW, Chew SJ, Capps S, Pedroza L, Chu ESS, Aeschbach J, Kaufman HE: Correlation of in-vivo confocal microscopy with scanning electron microscopy in demonstrating penetration of the eye by an organic foreign body. *Scanning* 14(suppl II), 44–45 (1992)

Binder PS: Cryotherapy for medically unresponsive *Acanthamoeba* keratitis. *Cornea* 8, 106–114 (1989)

Cavanagh HD, Jester JV, Essepian J, Shields W, Lemp MA: Confocal microscopy of the living eye. *CLAO J* 16, 65–73 (1990)

Cavanagh HD, Petroll WM, Alizadeh H, He Y, McCulley JP, Jester JV: Clinical and diagnostic use of in vivo confocal microscopy in patients with corneal diseases. *Ophthalmology* 100, 1444–1454 (1993)

Chew SJ, Beuerman RW, Assouline M, Kaufman HE, Barron BA, Hill JM: Early diagnosis of infectious keratitis with in vivo real time confocal microscopy. *CLAO J* 18, 197–201 (1992a)

Chew SJ, Beuerman RW, Kaufman HE: The tandem scanning confocal microscope as a clinical diagnostic tool in ophthalmology. *Scanning* 14(suppl II), 45–46 (1992b)

Chew SJ, Beuerman RW, Kaufman HE: In vivo assessment of corneal stromal toxicity by tandem scanning microscopy. *Lens Eye Toxic Res* 9, 275–292 (1993)

Inoue S: Foundations of confocal scanned imaging in light microscopy. In *Handbook of Confocal Microscopy* (Ed. Pawley J). Plenum Publishers, New York (1992) 1–14

Jester JV, Cavanagh HD, Lemp MA: In vivo confocal imaging of the eye using tandem scanning confocal microscopy. In *Proceedings of the 46th Annual Meeting of the Electron Microscopy Society of America* (Ed. Bailey GW). EMSA, San Francisco (1988) 56–67

Jester JV, Cavanagh HD, Lemp MA: Confocal microscopic imaging of the living eye with tandem scanning confocal microscopy. In *Noninvasive Diagnostic Techniques in Ophthalmology* (Ed. Masters BR). Springer-Verlag, New York (1990) 172–188

Jester JV, Petroll WM, Feng W, Essepian J, Cavanagh HD: Radial keratotomy. I. The wound healing process and measurement of incisional gape in two animal models using in vivo confocal microscopy. *Invest Ophthalmol Vis Sci* 33, 3255–3270 (1992a)

Jester JV, Petroll WM, Garana RMR, Lemp MA, Cavanagh HD: Comparison of in vivo and ex vivo cellular structure in rabbit eyes detected by tandem scanning microscopy. *J Microsc* 165, 169–181 (1992b)

Katakami C, Sahori A, Kazusa R, Yamamoto M: Keratocyte activity in wound healing after epikeratophakia in rabbits. *Invest Ophthalmol Vis Sci* 32, 1837–1845 (1991)

Kaufman HE: The correction of aphakia. *Am J Ophthalmol* 89, 1–10 (1980)

Kaufman SC, Beuerman RW, Kaufman HE: Diagnosis of advanced Fuchs' endothelial dystrophy with the confocal microscope. *Am J Ophthalmol* 116, 652–653 (1993)

Kay EP, Gu X, Ninomiya Y, Smith RE: Corneal endothelial modulation: A factor released by leukocytes induces basic fibroblast growth factor that modulates cell shape and collagen. *Invest Ophthalmol Vis Sci* 34, 663–672 (1993)

Klyce SD, Beuerman RW: Structure and function of the cornea. In *The Cornea* (Eds. Kaufman HE, Barron BA, McDonald MB, Waltman SR). Churchill Livingstone, New York (1988) 3–54

Koester CJ: A comparison of various optical sectioning methods: The scanning slit confocal microscope. In *Handbook of Confocal Microscopy* (Ed. Pawley J). Plenum Publishers, New York (1992) 207–216

Lam DSC, Chew SJ, McDonald MB, Beuerman RW: Modulation of corneal wound healing after excimer laser keratoplasty with muscarinic and histamine receptor antagonists. *Invest Ophthalmol Vis Sci* 34(ARVO suppl), 705 (1993)

Masters BR, Kino GS: Confocal microscopy of the eye. In *Noninvasive Diagnostic Techniques In Ophthalmology* (Ed. Masters BR). Springer-Verlag, New York (1990) 152–171

McDonald MB, Chew SJ, Ahmed S, Varnell R, Kaufman HE, Beuerman RW: In vivo evaluation of ocufen and corticosteroid treatment following excimer laser PRK using real-time confocal microscopy. *Invest Ophthalmol Vis Sci* 33(ARVO suppl), 1235 (1992)

Michels RG, Kenyon KR, Maumenee AE: Retrocorneal fibrous membrane. *Invest Ophthalmol* 11, 822–831 (1972)

Rozsa AJ, Guss RB, Beuerman RW: Neural remodeling following experimental surgery of the rabbit cornea. *Invest Ophthalmol Vis Sci* 24, 1033–1051 (1983)

Schanzlin DJ, Jester JV, Kay ED: Cryolathe corneal injury. *Cornea* 2, 57–68 (1983)

Underwood LW, Kelly C, Chew SJ, Beuerman RW: In vivo monitoring of corneal nerve injury by tandem scanning confocal microscopy. *Soc Neurosci Abstr* 18, 967 (1992)

Unterman SR, Beuerman RW, McDonald MB, Kaufman HE: Clinical healing after epikeratophakia. In *Healing Processes in the Cornea, Advances in Applied Biotechnology Series, vol. 1* (Eds. Beuerman RW, Crosson CE, Kaufman HE). Portfolio Publishing Company, The Woodlands, Texas (1989) 213–224

Werblin TP, Klyce SD: Epikeratophakia: The surgical correction of myopia. I. Lathing of corneal tissue. *Curr Eye Res* 1, 591–597 (1982)

Zavala EY, Binder PS, Deg JK, Baumgartner SD: Refractive keratoplasty: Lathing and cryopreservation. *CLAO J* 11, 155–162 (1985)